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Assessment of Test Portion Sizes after Sample Comminution with Liquid Nitrogen in an Improved High-Throughput Method for Analysis of Pesticide Residues in Fruits and Vegetables

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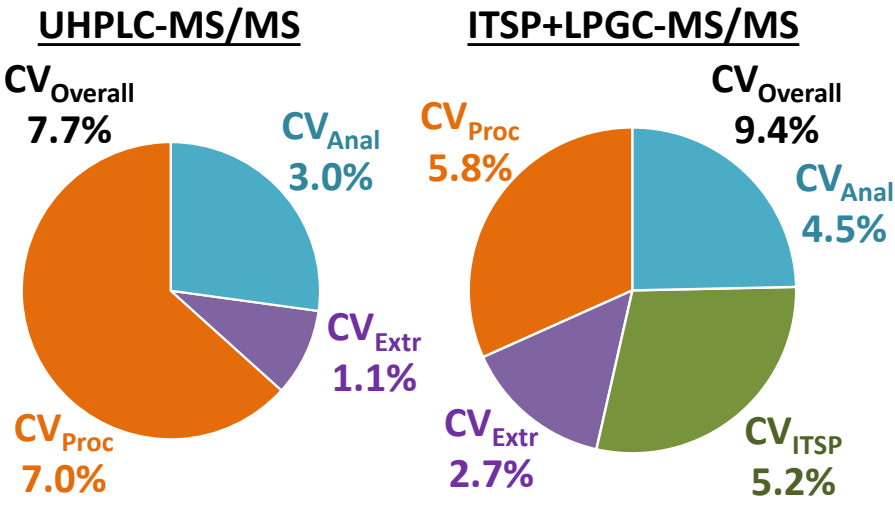
Note: The authors declare no competing financial interest.

SAFETY NOTE: The manufacturer of the sample processing device used in this study did not design nor test their product for use with liquid nitrogen. Appropriate care should be taken to avoid liquid nitrogen contact with skin and possible asphyxiation due to nitrogen displacement of oxygen in an enclosed breathing space.

Table of contents graphic image:



Abstract

In this study, sample processing of bulk commodities using an efficient one-step comminution procedure with liquid nitrogen (LN_2) was devised and assessed in the analysis of pesticide residues in fruits and vegetables. The LN_2 was added to the fresh samples from a tank by opening a valve, and the standard food chopper was kept in a laboratory hood to reduce safety risks. Test portions of 4 replicates each of 0.25, 0.5, 1, 2, 5, 10, and 15 g were taken from 8 fruits and vegetables (tomato, squash, broccoli, apple, grape, peach, green bean, and cucumber) individually comminuted with LN_2. For comparison without comminution, similar test portions of a reconstituted freeze-fried certified reference material of pesticides in cucumber were also analyzed by the same method. More than 100 pesticides were monitored by both ultrahigh-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) and instrument-top sample preparation (ITSP) + fast low-pressure gas chromatography (LPGC)-MS/MS. A new version of QuEChERS-based sample preparation was followed in which 5 mL 4/1 (v/v) acetonitrile/water per g sample is used for extraction, 200 µL initial extract is quickly evaporated, reconstituted in water, and ultra-centrifuged for UHPLC-MS/MS analysis. For ITSP+LPGC-MS/MS, another portion of the initial extract undergoes salt-out partitioning with 4/1 (w/w) anh. $MgSO_4$/NaCl and the upper layer extract is transferred to an autosampler vial for automated cleanup and analysis in parallel. Quality control spikes were made during the comminution, extraction, cleanup, and analyses steps to isolate and estimate the individual and overall measurement uncertainties of the approach. Recommended test portion size is 2 g for routine monitoring by this approach, but results demonstrated that subsamples as low as 0.5 g typically gave overall biases and RSDs <10% for nearly all pesticides, commodities, and methods, which is 3-5% lower than previously evaluated sample processing and analytical methods. This approach can be used to improve data quality, laboratory efficiency, and sample throughput in routine monitoring programs for regulatory, risk assessment, and other purposes.

Keywords: Sample Processing; Comminution; Liquid Nitrogen; Measurement Uncertainty; Pesticides Analysis; Fruits and Vegetables

■ INTRODUCTION

Just as a chain is only as strong as its weakest link, the sample processing step in the analysis

of pesticide residues in foods is as equally important as any other step in the overall method, but this essential step is usually ignored among monitoring laboratories and researchers. Despite its importance as a significant source of error, the comminution step, including the taking of test portions, is rarely included in analytical method validation or routine quality control (QC) protocols. Recently, a series of articles have been published in an attempt to change that oversight among analytical chemists in the pesticide residue analysis community.[1-10]

Regulatory purposes require that bulk commodities typically >1 kg be collected and processed to yield pesticide determinations that accurately correspond to the original sample lot (*e.g.* shipment or warehouse).[11-14] Thus, the comminution procedure must effectively produce test portions for analyses that are representative of the collected bulk sample. Prior to ≈1990, the typical test portion size taken for pesticide residue analysis in foods was 50–100 g,[15] which was generally decreased to 25–30 g during the 1990s.[16,17] With the advent of QuEChERS in the 2000s, the test portion size further decreased to 10–15 g, but QuEChERS was introduced with a warning that greater care must be taken in the comminution step to achieve representative accuracy in the results due to the smaller subsample size.[18] Thorough comminution is also critically important to achieve acceptable extraction efficiency by shaking with solvent in QuEChERS rather than blending as in previous methods.

Despite the greater sample processing variability introduced due to the smaller test portion size of QuEChERS and similarly miniaturized methods,[18-20] few if any labs took precautions to validate their comminution step or include it among routine QC practices. Meanwhile, in accordance with ISO 17025 standards,[21] proficiency testing at considerable effort and expense has become an accepted practice to evaluate laboratory and method performance, which only entails the analysis of pre-comminuted samples, thereby shirking the equally essential sample processing step conducted by every laboratory. Even the gold standard evaluations of methods using certified reference materials (CRMs) and/or inter-laboratory collaborative studies avoids the sample comminution step by providing pre-blended test samples for analysis. The current practice of willful neglect to consider sample processing as part of the overall method give a new ironic meaning to the expression of "blind" sample analysis.

Defenders of the status quo may say that the comminution steps have already been validated, but previous studies actually show that quality of sample processing is highly dependent on techniques, devices, analytes, and matrices, often contributing >50% to the overall measurement

uncertainty in real-world analysis.[1,2,4-9,16,17] By that logic, proficiency testing is unnecessary because analytical methods in common use have already been extensively validated by many analysts in many laboratories worldwide, including via frequent proficiency testing analyses for decades.[22,23] Yet, very few validation studies of sample processing have been conducted by comparison. Despite this reality, regulators and analytical chemists continue to ignore the comminution step in proficiency testing, method validation, and routine QC practices.

The argument that it is too difficult or expensive to evaluate sample processing is unfounded considering the high costs of existing quality assurance practices, including laboratory accreditation, proficiency testing, laboratory information management systems, and inefficiencies in general. Even so, the cost argument is specious because periodic assessment of sample processing is free when replicate test portions of incurred samples already have to be re-analyzed for other purposes.[1] Also, it is very simple to spike commonly available (inexpensive) pesticides into bulk samples during the comminution step rather than the extraction step.[2,4-7,9]

Perhaps the most prominent reason that sample processing is frequently ignored by many investigators, especially egregious in the case of miniaturized analytical methods,[3,8] is that they cannot overcome the technical challenge to devise a practical and fast approach that yields <2 g test portions to accurately represent the original bulk commodity. Riter *et al.,*[6,7] validated a 2-step approach using relatively expensive cryogenic milling devices with limited sample throughput to achieve acceptably representative test portions <0.5 g for certain (but not all) pesticide/matrix combinations. Lehotay *et al.*[4,5] using the same or similar means could not match that feat for a wider range of pesticides and commodities, and they concluded that use of 2-5 g test portions were feasible in a straight-forward single step approach.

In a pioneering study, Roussev *et al.*[3] described the use of liquid nitrogen (LN_2) within a standard food processing device for cryogenic sample comminution rather than the more common use of dry ice. Although LN_2 was used in the specialized devices reported previously,[4-7] the samples were not directly exposed to the LN_2 in those cases. In practice, maximum sample sizes were only ≈20 g using the cryogenic mills, thereby necessitating a 2-step approach. Roussev *et al.* did not explore the acceptability of test portions <10 g in their study,[3] but if a straight-forward single-step comminution method can be conducted using inexpensive tools to achieve ≤1 g acceptably representative test portions, then miniaturized methods can be implemented to greatly increase sample throughput in routine pesticide residue monitoring labs

at reduced costs. The untrue argument that validation of the comminution step increases laboratory expenses would be undermined by this outcome, because even if it were true, the cost savings in routine analysis could be applied to QC assessment of sample processing.

With this outcome in mind, the aim of this study was to evaluate the ability of LN_2 comminution to provide an efficient and effective 1-step process to prepare representative test portions from bulk fruit and vegetable samples for high-throughput analysis. Another objective was to devise safe and efficient operations using LN_2-comminution with a typical commercial food processor. An important goal was to determine the smallest test portion amount subsampled from a 500 g bulk sample that maintained acceptable accuracy in the analytical results. An improved QuEChERS-based sample preparation method and optimized analytical methods using ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) and automated instrument-top sample preparation (ITSP) + fast low-pressure gas chromatography – tandem mass spectrometry (LPGC-MS/MS) were also to be assessed in the application.

■ MATERIALS AND METHODS

Chemicals, reagents, and solutions.

Pesticide standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg; Germany), Sigma-Aldrich (St. Louis, MO; USA), ChemService (West Chester, PA; USA), or the Environmental Protection Agency's National Pesticide Repository (Fort Meade, MD; USA). Atrazine-d5 and fenthion-d6 were from C/D/N Isotopes (Pointe-Claire, Quebec; Canada), *p*-terphenyl-d14 was from AccuStandard (New Haven, CT; USA), and ^{13}C-phenacetin came from Cambridge Isotope Laboratories (Andover, MA; USA). Anhydrous magnesium sulfate (anh. $MgSO_4$), sodium chloride (NaCl), 3-ethoxy-1,2-propanediol, L-gulonic acid γ-lactone, D-sorbitol, shikimic acid, toluene, ammonium formate, and formic acid originated from Sigma-Aldrich. Premade mixtures of 2 g 4/1 (w/w) anh. MgSO4/NaCl in 15 mL polypropylene centrifuge tubes were purchased from UCT (Bristol, PA; USA) and Agilent (Little Falls, DE; USA). Deionized water (18.2 MΩ-cm) came from a Barnstead/Thermolyne (Dubuque, IA; USA) E-Pure Model D4641, and HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were from Fisher Scientific (Pittsburgh, PA; USA). Mini-solid-phase extraction (SPE) cartridges for

ITSP containing 45 mg anh. $MgSO_4$/C18/primary secondary amine (PSA)/CarbonX (20/12/12/1, w/w/w/w) were purchased from ITSP Solutions (Hartwell, GA; USA). Liquid nitrogen (LN_2) was available in the in-house system at the USDA Eastern Regional Research Center delivered in bulk by Air Liquide (Norristown, PA; USA).

The Certified Reference Material (CRM) of pesticides in dried cucumber (ERM- BC403) was from European Reference Materials (Geel, Belgium) obtained via Sigma-Aldrich in the USA, and > 2 lbs. samples of cherry tomato, summer squash, broccoli, apple, grape, peach, green bean, and cucumber were purchased at local supermarkets.

Stock solutions of pesticides at ≈2,000 ng/µL were typically prepared in toluene and aliquoted into mixtures in MeCN for different QC purposes, with analyte lists defined in Tables S1 and S2 (supplemental). As displayed in Figure 1, a similar design of the study was followed as conducted previously,[4] in which different QC spikes were made during each step in the analytical method using 4 replicates of different test portion sizes (15, 10, 5, 2, 1, 0.5, and 0.25 g) of each commodity. For sample processing (QC_{Proc}), a mixture of 18 pesticides was prepared at 55.6 ng/µL. For extraction (QC_{Extr}), 5 ng/µL each of the internal standards (atrazine-d5, fenthion-d6, and pyridaben-d13) were prepared, which was diluted to 1 ng/µL for addition to 0.5 g and 0.25 g sample test portions. In the case of automated ITSP cleanup prior to LPGC-MS/MS analysis (QC_{ITSP}), a mixture of 0.67 ng/µL each of carbophenothion, procymidone, and piperonyl butoxide was prepared. For analysis (QC_{Anal}), 0.068 ng/µL of ^{13}C-phenacetin was prepared in water for use in UHPLC-MS/MS, and for LPGC-MS/MS, 0.55 ng/µL *p*-terphenyl-d14 was included in the analyte protectant (AP) solution of 30 µg/µL 3-ethoxy-1,2-propanediol, 1.5 µg/µL shikimic acid, 3 µg/µL each of L-gulonic acid γ-lactone and D-sorbitol, and 2.5% formic acid in 4/1 (v/v) MeCN/water.

Incurred pesticides were also determined in the samples to assess bias and precision in the analyses, and a mixture of 77 pesticides at 10 ng/µL was prepared for eventual use in 6-point calibration standards (0, 2, 8, 32, 128, and 512 ng/g equivalents, including QC_{Proc} and QC_{ITSP} analytes, plus 100 ng/g equivalents of QC_{Extr}, and QC_{Anal} analytes). Separate calibration standards were prepared for the analysis of the CRM sample ranging from 0, 0.25, 0.5, 1, 1.5, and 2 times (X) the certified concentration for each pesticide. The certified concentrations of the pesticides in the reconstituted cucumber CRM sample consisted of 64 ng/g acetamiprid, 639 ng/g azoxystrobin, 74 ng/g carbendazim, 64 ng/g chlorpyrifos, 45 ng/g cypermethrin, 51 ng/g

diazinon, 31 ng/g endosulfan I, 54 ng/g fenitrothion, 44 ng/g imazalil, 627 ng/g imidacloprid, 570 ng/g iprodione, 52 ng/g malathion, 59 ng/g methomyl, 61.1 ng/g tebuconazole, and 56 ng/g thiabendazole. Endosulfan II and endosulfan sulfate were also included in the calibration standards with 1X at 31 ng/g.

Sample processing.

In the case of the CRM, the instructions in the certificate of analysis were followed by adding 72 g of deionized water each to 2 bottles of the dried cucumber, which were both vortexed for 2 min to mix well and then combined into a 250 mL wide-mouth Teflon bottle. The reconstituted sample was re-vortexed each time as 4 replicates each of 0.25, 0.5, 1, 2, 5, 10, and 15 g test portions were weighed for analysis in the design shown in Figure 1.

The bulk raw commodities were freshly purchased and kept at room temperature prior to processing with LN_2 using a Robot Coupe (Ridgeland, MS; USA) Blixer 2 with a fine serrated s-shaped blade. All 8 commodities (stems removed) were processed by a pair of technicians, and two stainless steel bowls with lids and blades were alternated for use, which were dried well and pre-chilled for 5-10 min in a -80ºC freezer before being inserted with blade into place on the chopper motor. In the cases of cherry tomatoes, grapes, and green beans, 500 g portions of the bulk raw samples at room temperature were added directly to the mixing bowl (a larger bowl would have been more conducive for 1 kg samples). The peach (pits removed), broccoli, apple, cucumber, and squash were first cut with a knife into ≈2 cm^3 chunks before the 500 g was added (step 1 in Figure 1). Then in each case, 900 µL of the QC_{Proc} solution was pipetted onto a few sample surfaces in the bowl (inhomogeneous initial distribution), and the lid and LN_2 tubing was put into place (step 2).

Based on preliminary tests using a Dewar, ≈1.5 L of LN_2 was sufficient to process 500 g fruit or vegetable sample added at room temperature to the pre-cooled containers, which matched previous findings.[3] Watery fruits required more LN_2 and green vegetables needed less. Pre-chilling of the bowls was ultimately deemed not to be worthwhile, but it did lead to slightly reduced sample processing time and LN_2 consumption.

Figure S1-A (supplemental) displays a photograph of the setup in which the food processor is contained in a closed laboratory fume hood and the LN_2 is fed through tubing from a 160 L tank. In this way, the hood practically eliminates asphyxiation risk in the lab due to excess N_2 gas, and

human exposure to the LN_2 is avoided via use of plumbing and valves rather than pouring the LN_2 into the container from a Dewar by hand. The latter practice has greater safety risk and requires substantial physical strength, whereas the opening and closing of a valve is safe and easy. Even so, the operator wore cryogenic safety gloves, a coat, and face shield to reduce the chance of exposure. The Blixer scraper arms had been removed from the polycarbonate lids to provide the opening for the LN_2 tubing, and nine ≈4 mm holes were drilled toward the back of each lid to help release the N_2 gas when LN_2 was added to the bowls.

The tank valve was opened to release the LN_2 into the bowl (steps 3-4 in Figure 1), and in ≈10 s increments, the pulse button on the motor was pressed in ≈2 s bursts to initiate the mixing process and avoid locking of the blade within the frozen sample. The condition of the sample within the bowl was assessed by sounds of crackling during the ≈10 s intervals and impacts against the stainless steel container during the bursts of the blade. It typically took ≈45 s for clear indication that the sample no longer liquefied when the motor was pulsed. At that point, the chunks of sample were not "sliced" by the blade, but they were shattered as if struck by a hammer. When the distinct difference in the sound was heard, the motor "on" button was pressed for the blade to run continuously as the LN_2 was still being added. After ≈30 s, the LN_2 valve was closed, and another ≈30 s was given for the LN_2 to dissipate before motor "off" button was pressed. Operator experience was a key factor in being able to conduct LN_2 comminution in this study, and several trial-and-error practice attempts were needed for the operator to feel comfortable in devising and performing the final protocol.

The container was removed from the motor, followed by removal of the lid and blade from the bowl, which was taken immediately to a top loading balance. A very fine frozen powder was produced that did not stick to surfaces for ≈10 min, which made it easy for the second technician to transfer the comminuted sample first to pre-cooled 1 L glass jars using a cold metal spoon. The first technician could then wash, dry, and place the Blixer container in the freezer (if needed) for re-use, and take the other container from the freezer for the next commodity. Meanwhile, the second technician employed pre-chilled plastic spatulas as described by Riter *et al.*[6,7] to transfer 4 replicates each of 0.25, 0.5, 1, 2, 5, 10, and 15 g test portions (in that order) following the design shown in Figure 1 (step 5). For the 0.25 g samples, 2 mL polypropylene (PP) mini-centrifuge tubes were used, 0.5–2 g portions were weighed into 15 mL PP centrifuge tubes, 5 g into 50 mL PP tubes, and the 10 and 15 g into 250 mL PP centrifuge bottles. The

technician ensured that all containers were sealed well for storage in a -80ºC freezer until the day of sample preparation and analysis (within 4 weeks).

Sample preparation.

After the pre-weighed test portions were given ≈15 min to thaw (or right away for the CRM), sample preparation consisted of the following steps as outlined in Figure 1: 6) QC_{Extr} solution was added to yield 100 ng/g each of the internal standards (int. stds.) to all samples (not the reagent or cucumber blanks), and each tube/bottle was briefly vortexed to mix the spike into the matrix; 7) 5 mL 4/1 (v/v) MeCN/water per g test portion was added to each sample (*e.g.* 75 mL for 15 g sample and 1.25 mL for 0.25 g); a Glas-Col (Terre-Haute, IN; USA) platform pulsed vortexer at maximal shaking and 80% pulsation setting was used for 10 min sample extractions; and a Kendro (Osterode, Germany) Sorvall Legend RT swinging bucket centrifuge fitted with different adapters for the different centrifuge tubes/bottles was used for centrifugation at room temperature for 5 min and 3711 rcf.

For UHPLC-MS/MS: 8) 200 µL of each extract was transferred to 2 mL PP mini-centrifuge tubes, which were placed within 15 mL glass centrifuge tubes fitted with paper clips held by rubber bands into a Zymark (Hopkinton, MA; USA) TurboVap LV, as shown in Figure S1-B (supplemental), and evaporated to just dryness (≈5 min) using 10 psi N_2 flow setting and 40ºC water bath temperature; 9a) 750 µL water plus 50 µL ^{13}C-phenacetin solution (QC_{Anal} for LC) to yield 100 ng/g equivalent sample concentration was added to all tubes, plus 15.4 µL MeCN for the samples; 10a) the tubes were centrifuged for 5 min at 4ºC and 12,000 rcf using a Tomy Seiko (Tokyo, Japan) MTX-150 mini-centrifuge, and 525 µL final extracts (avoiding possible precipitant) were pipetted into 0.8 mL PP vials for placement in the UHPLC autosampler tray.

For ITSP+LPGC-MS/MS: 9b) 10 mL of extract was transferred from the 2–15 g sample supernatants from Step 7 into 15 mL PP centrifuge tubes containing 2 g 4/1 (w/w) anh. $MgSO_4$/NaCl, and likewise, 5 mL was pipetted into 1 g of salts for the 1 g test portions, 2 mL into 0.4 g salts for the 0.5 g sample amounts, and 1 mL into 0.2 g salts for the 0.25 g portions; the tubes with salts were shaken for 1 min and centrifuged for 3 min using the same devices and settings as in step 7; 10b) 800 µL of the upper layer extracts was transferred to amber glass autosampler vials (except for the 0.25 g test portions, which entailed 600 µL extract + 200 µL

MeCN), plus 30 µL of the QC_{ITSP} solution (22.5 µL for 0.25 g extracts) was added to yield 100 ng/g sample equivalent; and 12b) 10 µL of AP/QC_{Anal} for GC solution was added into 300 µL glass inserts within another set of amber glass autosampler vials, plus 25 µL MeCN in the case of samples, which were capped with MicroSolve (Leland, NC; USA) cat. # 9502S-E-3XB slit-septa caps. Both sets of vials were loaded onto the robotic trays for automated ITSP+LPGC-MS/MS analysis (11b), in which the ITSP cleanup step entailed 300 µL of the extract being passed through the 45 mg mixed QuEChERS sorbent cartridges at 2 µL/s into the receiving vials containing the glass inserts. The cleanup was performed just-in-time for injection in parallel as the previous cleaned-up sample was being analyzed via LPGC-MS/MS as described previously.[4,5,24-26]

Preparation of calibration standards.

Both sets of vials were weighed before and after ITSP to assess the consistency of the volumes as calculated from the density of MeCN. On average, final ITSP-eluted extracts were ≈220 µL (223.7 ± 9.9 in the study, n ≈ 300), which corresponded to ≈55 mg equivalent sample (4 mL MeCN per g sample was used for extraction). In the case of UHPLC-MS/MS, initial extracts were ≈0.17 g/mL accounting for the water in the samples, thus 200 µL corresponded to ≈34 mg equivalent sample. These amounts were used in calculations to prepare the calibration standards. A 4.5 g amount of water was used as a reagent blank and source for preparation of reagent-only (RO) calibration standards in each experiment. In the CRM analysis, 5 g of comminuted organically-grown cucumber was also used as a source for matrix-matched (MM) calibration standards. The sample preparation protocol for 5 g test portions was followed in both cases, and 6 aliquots each were taken from the same extract for addition of pesticides to yield the calibration standards at 0, 2, 8, 32, 128, and 512 ng/g (or 0, 0.25, 0.5, 1, 1.5, and 2X CRM levels) with QC_{Anal} and QC_{Extr} always kept at 100 ng/g. In UHPLC-MS/MS, 15.4 µL of calibration preparation solutions were added rather than MeCN to the RO and/or MM extracts in Step 6a, and similarly in Step 9b for LPGC-MS/MS, 25 µL of the same 6 calibration preparation solutions were added in place of MeCN. In the latter case, a second set of calibration standards using 220 µL MeCN + 25 µL calibration preparation solutions + 10 µL AP/QC_{Anal} solution were also analyzed in every sequence to compare with the standards prepared from the reagent blank after the ITSP cleanup step. In both LC and GC, the calibration standards were interspersed

among the samples within the analytical sequences.

UHPLC-MS/MS and ITSP+LPGC-MS/MS analyses.

ITSP cleanup was performed using a Gerstel (Linthicum, MD; USA) MPS-3 robotic autosampler, as shown in Figure S1-C (supplemental), controlled by Maestro software. Final extracts were immediately analyzed after automated ITSP by LPGC-MS/MS using an Agilent (Little Falls, DE, USA) 7890A gas chromatograph and 7010 triple quadrupole mass spectrometer controlled by MassHunter software. Separation of the analytes was performed by means of a Restek (Bellefonte, PA; USA) LPGC Pesticides Kit (cat. # 574262), which consisted of a 5 m, 0.18 mm i.d. uncoated capillary restrictor at the inlet coupled to a 15 m, 0.53 mm i.d., 1 μm film thickness Rtx-5MS analytical column plus an extra 1 m integrated uncoated 0.53 mm i.d. capillary for connection at the transfer line. Both paired-column dimensions were entered into the software for electronic flow control with vacuum outlet, but the analytical column remains fully under vacuum and only the restrictor capillary mattered in the software calculations. A standard Agilent split-splitless inlet containing a Restek Topaz low-pressure drop Precision liner with glass wool (cat. # 23309) was used for 4 μL injections at 280°C in switched splitless mode for 3 min, when 3 mL/min septum purge and 25 mL/min split vent flows were initiated. Oven temperature started at 80°C for 1 min when it was raised at 45°C/min to 320°C, where it was held for 3.7 min giving a total of 10 min. High purity helium was used as the carrier gas, and a ramped flow rate was applied starting at 2.25 mL/min for 3 min when it was lowered to 1.5 mL/min for the remaining 7 min. The transfer line and ion source were kept at 280°C and 320°C, respectively, and electron ionization was -70 eV and 100 μA filament current. Ion transitions were analyzed using dynamic multiple reaction monitoring (MRM), and retention times (t_R) along with different MS/MS conditions for the analytes are listed in Table S1 (supplemental).

For UHPLC-MS/MS, a Shimadzu (Columbia, MD; USA) Nexera X2 liquid chromatograph coupled to a Sciex (Foster City, CA; USA) 6500 triple quadrupole linear ion trap mass spectrometer was used for analysis. Sciex Analyst software was used for instrument control and MultiQuant for data processing. The analytical column was a Waters (Milford, MA; USA) BEH C18 of 100 mm, 2.1 mm i.d., and 1.7 μm particles fitted with a 5 mm pre-column of the same i.d. and stationary phase material. Column temperature was 40°C. Mobile phases

consisted of: (A) 95/2.5/2.5 (v/v/v) water/MeCN/MeOH; and (B) 1/1 (v/v) MeCN/MeOH, both including 0.1% formic acid and 20 mM ammonium formate. Flow rate was 0.5 mL/min, and the gradient was 95% A for 0.25 min, linearly ramped to 100% B over 7.5 min, and held for 2 min (until 9.75 min). Return to starting conditions was made over 0.75 min and 4.5 min were given for re-equilibration. Injection volume was 10 µL. Scheduled MRM was used with electrospray ionization source temperature of 450°C, ±4500 V ionspray voltage, and other conditions listed in Table S2 (supplemental).

Data processing and calculations of uncertainties.

Summation function integrated peak areas were used for quantification on both instruments as previously described.[25] For ITSP+LPGC-MS/MS, analyte concentrations were determined vs. atrazine-d5 using 1/x weighted quadratic calibration curves, except for chlorothalonil which used linear calibration without a weighting factor (the same as UHPLC-MS/MS quantifications were performed in all cases). Peak areas were also assessed for bias and precision in the experiments without use of calibration or normalization to int. stds.

As detailed previously,[4,5] RSD refers to measured results from experiments and CV designates the uncertainty component calculated from the RSDs. Fundamentally, a sum of squares relationship is used to make the uncertainty assessments in this study:

$$CV^2_{Overall} = CV^2_{Proc} + CV^2_{Extr} (+ CV^2_{ITSP}) + CV^2_{Anal} = RSD^2_{Proc}.$$

CV_{Anal} equals RSD_{Anal} of ^{13}C-phenacetin in UHPLC-MS/MS from the RO standards (to minimize contributions from matrix effects), and for LPGC-MS/MS, RSD_{Anal} of *p*-terphenyl-d14 from MeCN-only calibration standards is used to measure CV_{Anal} (to avoid the 4.4% RSD volume differences from the ITSP step as well as matrix effects). To isolate the cleanup component in ITSP+LPGC-MS/MS: $CV_{ITSP} = \sqrt{(RSD^2_{ITSP} - RSD^2_{Anal})}$. RSD_{ITSP} was the average of the three QC_{ITSP} analytes within a sequence, independent of test portion size since QC_{ITSP} spikes were made to fixed volumes of extracts. Similarly, $CV_{Extr} = \sqrt{(RSD^2_{Extr} - RSD^2_{ITSP})}$ for ITSP+LPGC-MS/MS and $CV_{Extr} = \sqrt{(RSD^2_{Extr} - RSD^2_{Anal})}$ in UHPLC-MS/MS, independent of test portion sizes assuming scaling of the method makes no difference (this facet was evaluated by also calculating CV_{Extr} for each subsample amount). Only atrazine-d5 was used for RSD_{Extr} because fenthion-d6 and pyridaben-d13 degraded in some matrices (as before).[4] Lastly, $CV_{Proc} = \sqrt{(RSD^2_{Proc} - RSD^2_{Extr})}$, which was calculated for each test portion size for each

matrix and analyte as well as in overall calculations. Theoretically, the same calculated measurement uncertainty values for sample processing should be obtained for the same analyte and commodity in both ITSP+LPGC-MS/MS and UHPLC-MS/MS, but the results can only be treated as estimates due to practical limitations and variabilities involved to conduct such a study. Many pesticides and matrices were compiled in the CV_{Proc} component to increase reliability of the estimations.

■ RESULTS AND DISCUSSION

Analysis of the cucumber CRM

In an experiment to assess the effect of test portion size independent of the LN_2 comminution component in the overall method, a CRM of dried cucumber was analyzed for the many pesticides listed with their certified concentrations in Materials and Methods. Two bottles of the commercial CRM were purchased, both were reconstituted with 72 g water as described in the instructions, and combined. The procedure in Figure 1 starting with Step 6 was followed except using pipets rather than spatulas for transfers, as also detailed in Materials and Methods.

Table 1 contains the determined concentrations in the CRM for both the UHPLC-MS/MS and ITSP+LPGC-MS/MS analyses independent of the test portion size (n = 28). In terms of precision, the results were excellent even when including the 0.25 and 0.5 g test portions, with all RSDs ≤7% and averages of 3.2% in UHPLC-MS/MS and 5.3% in ITSP+LPGC-MS/MS. Similarly, comparison of the determinations for the same pesticides analyzed by both instruments averaged merely 2% difference with respect to azoxystrobin (0.4%), imazalil (4%), iprodione (0.3%), malathion (2%), and tebuconazole (4%). Diazinon and thiabendazole gave 14-15% differences, but diazinon results by LC are not as reliable as those by GC, and vice versa for thiabendazole.

In terms of trueness (recovery) of the determinations vs. the certified values, a clear but consistent bias occurred that requires explanation. In both LC and GC, 8 pesticides in each case gave 147% average recoveries with merely 4% RSD among them in LC and 11% in GC. Recoveries are known to be 100% for those and many other pesticides in several validation experiments, which leads to the conclusion that a 47% bias was introduced into the experiment.

The calibrations of balances, pipets, and calculations used to prepare solutions and conduct analyses were checked and re-checked, and no errors were found. Differences in calculated vs. actual g/mL equivalent sample in final extracts could not amount to more than a 5% bias, and such an error would have also occurred for spiked samples. Furthermore, the calibration curves gave no concerns at all concentrations, normalizations to an int. std. made little difference in the results, and QC_{Anal}, QC_{ITSP}, and QC_{Extr} spikes gave 100-110% recoveries with ≤11% RSD (see Table 2). Lastly, matrix effects were minimal in both the LC and GC analyses for nearly all analytes as shown in Figure S2 (supplemental), except for imazalil, azoxystrobin, tebuconazole, and diazinon in LC, but as already stated, the concentration determinations for those (and other) pesticides were very similar in both analytical methods.

All of these factors point to accurate analytical results for the CRM, and a possible reason for the bias is that the actual weights of the dried samples in the vials were not measured. The instructions stated that each vial contained "3.2 g dried cucumber," but the "description of the material" stated "approximately 3.2 g." This discrepancy may have been the source of the bias, but it does not matter in this study because precision (RSDs) was assessed directly and bias vs. test portion size was always relative to the 15 g results. Just for the sake of curiosity, recoveries normalized to 147% in the CRM led to 93-112% trueness for all analytes in both LC and GC except for carbendazim (121% in LC), diazinon (63% in LC and 83% in GC), imazalil (66% in LC and 72% in GC), and thiabendazole (79% in GC).

Figure 2 shows the difference in RSDs (upper plot) and relative bias (lower) vs. test portion amounts for the CRM (no LN_2 comminution) compared with the LN_2-comminuted cucumber sample. The reconstituted CRM subsamples from 1–15 g for the 16 pesticides determined by LC and/or GC analysis with or without use of an int. std. gave an average of 2.7% RSD, whereas the 21 pesticides incurred and spiked in cucumber analyzed by the same methods averaged 5.7% in the range of 0.5 g to 15 g test portions. The 3% RSD difference between the store-bought and CRM cucumber results can be attributed to the LN_2 comminution step, which corresponds to CV_{Proc} = 5% for cucumber as calculated by: $\sqrt{(5.7^2 - 2.7^2)}$. Also, the ≈3% (absolute) increase in average RSDs in both cases at the 0.25 g test portion size demonstrates the higher degree of imprecision as the subsample gets too small.

In terms of bias relative to results for the 15 g subsamples, Figure 2 (bottom) shows how no significant bias occurred overall for the LN_2-comminuted cucumber sample with respect to test

portion size, but a slight relative bias of 2.4% resulted in the 1–10 g range of CRM test portions. No notable differences were observed when using an int. std. or not, except in the case of the CRM for the 0.25 and 0.5 g test portions in both LC and GC. A different diluted QC_{Extr} solution was used for those test portion sizes in the protocol, which likely caused the effect on that day. Otherwise, the CRM relative bias averaged 2.1% from 0.25–10 g (actually less considering that the 15 g subsample probably gave a slightly negative bias). The CRM instructions call for a minimum test portion of 2.5 g reconstituted sample, but the same quality of results was achieved using as little as 1 g in our method, or 0.5 g in the case of the LN_2-comminuted cucumber.

Analytical method considerations

The original QuEChERS version used previously followed by ITSP+LPGC-MS/MS and UHPLC-MS/MS did not easily allow test portions <1 g simply due to the logistics of pipetting such small volumes from the sample extracts.[4,5] Rather than the usual extraction ratio of 1 mL MeCN per g sample, this newer QuEChERS-based version calls for 5 mL extraction solvent per g test portion, which conforms with the QuEChERS-based sample preparation for analysis of veterinary drugs in foods for inclusion of an even broader range of analytes if needed.[27] The extra volume of extract makes subsequent transfers for both methods of analysis much easier for test portions as small as 0.25 g. Subsamples smaller than that led to insufficient extract for the ITSP step, unless perhaps a 96-well plate format is adopted. The protocol requires transfer of only the upper MeCN layer extract from the tube containing the salts, which becomes trickier as volumes decrease. Although 300 µL is the minimum extract needed for the ITSP step, the format using standard autosampler vials required >600 µL (800 µL was used for good measure) to avoid air bubbles in the 1 mL ITSP syringe. In the case of 0.25 g test portions, a small amount of lower phase (water) was sometimes unavoidably transferred along with the upper phase (MeCN) extract. Thus, sample homogenization is not the only reason that the degree of precision decreased for the 0.25 g subsamples as shown in Figure 2, but the greater difficulty to perform the miniaturized method, at least in the case of ITSP+LPGC-MS/MS, also contributed to the higher RSD.

This situation for the GC analysis of the 0.25 g subsample is readily apparent in Figure 3, which is a compilation of all incurred and spiked pesticides in all LN_2-comminuted matrices.

On average, a ≈13% negative relative bias occurred for the 0.25 g test portions in GC compared with less than ±5% relative bias in LC for any test sample amount. In ITSP+LPGC-MS/MS, the trend shown in Figure 3 of increasing average relative bias from 0% to ≈4% from 15 to 0.5 g was not solely due to test sample size. A clear trend was observed for some commodities (particularly grape and peach) that the analyte responses took up to 10 injections at the start of analytical sequences to reach a consistent response factor.

Undoubtedly, the effect was caused by "priming" of the LPGC-MS/MS system with APs and matrix components because those pesticides most severely affected, such as strobilurins (e.g. pyraclostrobin and azoxystrobin) and triazoles (e.g. tetraconazole), interact most strongly with active sites in the system.[28] All sequences began with 3 MeCN injections followed by the 0 and 2 ng/g calibration standards, then the first 15 g replicate followed by 10, 5, 2, 1, 0.5, and 0.25 g before repetition in that order of the 2nd through 4th sets of replicates with additional calibration standards and MeCN injections in between. Thus, the slight trend in relative bias in the GC results was an artefact of the analytical sequence due to stabilization of matrix effects, not due to LN_2-comminution. Another factor with this protocol is that the amounts of APs injected varied slightly in proportion to ITSP elution volumes, thus causing greater inconsistencies in matrix effects than would have occurred by adding the APs in the injection syringe. Even so, average relative biases for 1–15 g subsamples were small (<9%) in this study, depending slightly on normalization to atrazine-d5 or not (see Figures 2 and 3). Previous studies showed at least double the extent of relative biases up to 18% for 1 g subsamples with much clearer trends vs. test portion size.[4,5]

Results of the LN_2-comminuted samples

For the sake of those who may wish to process the results in different ways than presented in this paper, Tables S3 and S4 (supplemental) give the average concentrations or recoveries and their precision vs. atrazine-d5 for all incurred and spiked pesticides, commodities, and test sample sizes. As Figures 2 and 3 show, small differences with a few exceptions occur due to the int. std. Tables 1 and 2 provide the averages and (relative) standard deviations of those results for each commodity independent of test portion size. Although it was justifiable to limit the range to only include the results from the 1–15 g samples without use of the int. std., which gave better overall accuracy, all results were included to provide conservative estimations, not

best case scenarios. The effect of test portion sizes are presented and discussed separately.

With respect to incurred pesticides (Table 1), the UHPLC-MS/MS and ITSP+GC-MS/MS results for overlapping pesticides are in generally good agreement, as already mentioned about the CRM, except for some of the pesticides in grapes and peaches. Software and firmware updates of both the GC-MS/MS and robotic autosampler at that time led to an error in ITSP performance that required troubleshooting before the problem was corrected (the default setting for "touchdown current" was much too high). In grapes and peaches, different test portions were taken a month apart for GC and LC analyses, and sample storage and freeze/thaw effects probably led to the larger differences for cyprodinil, difenoconazole, fenbuconazole, and pyraclostrobin.

Otherwise, consistently comparable results with "quantidentifications" < 1 ng/g for many incurred pesticides were demonstrated for both methods. In regulatory monitoring, determinations < 10 ng/g fall below the typical minimum reporting level,[12] but <1 ng/g limits of quantification are appropriate for risk assessment purposes.[13] All pesticides found match those often reported elsewhere in the given commodities.[13] Despite higher variabilities of the incurred pesticide results, largely due to lower concentrations,[4] incurred and spiked pesticides were compiled together in the study to yield average RSD_{Proc} used for calculations of CV_{Proc}. Again, use of QC_{Proc} pesticides only would have been justifiable to show better method precision, but the goals of the study are better met by showing the real-world situation.

With respect to spiked pesticides, recoveries and RSDs are reported in Table 2 for each commodity with averages of each factor listed for all commodities beneath the QC_{Extr} analytes in the CRM column. Starting with QC_{Anal}, the differences from the 100% trueness result that would be expected arise from a combination that peak areas were normalized to atrazine-d5 (QC_{Extr}) and differing matrix effects of both QC_{Anal} standards as well as atrazine-d5. The same is true for all analytes in Table 2 except for atrazine-d5, which shows the recoveries without normalization to itself. In truth, atrazine-d5 is recovered ≈100% in the sample preparation method(s) in each commodity, and the differences shown from 100% result from matrix effects and differences in day-to-day pipetting to prepare solutions and extracts, in ITSP volumes (for GC), and in different amounts of water in the commodities that affect volumes of initial extract and the MeCN phase during the salt-out partitioning step. The addition of multiple QC analytes, such as the 3 pesticides averaged for QC_{ITSP}, help to reduce inaccuracies, but fenthion-

d6 and pyridaben-d13 were poorly detected in UHPLC-MS/MS and also degraded in nonacidic stored solutions and extracts to render them unserviceable in the study. Spinosad degraded in calibration solutions, and the UHPLC-MS/MS also lost sensitivity for parathion-methyl, diazinon, propargite, and pyriproxyfen, but this was unrelated to the method and rectified in subsequent studies.

When judging the QC_{Proc} recoveries and RSDs in Table 2 and elsewhere in this report, it must be kept in mind that unlike most studies in which the analytes are spiked into the test portions just prior to extraction, these pesticides were inhomogeneously added to the 500 g bulk samples prior to LN_2-comminution followed by storage at -80ºC for many weeks in some cases. Despite this, the recoveries for nearly all analytes were ≈100% with RSDs <10% even when including 0.25 g test portions. Azinphos-methyl is known to be a difficult analyte, but LC and GC yielded 100 ± 12 %recoveries for it overall. Chlorothalonil is another problematic pesticide, especially when using MeCN or acetone for extraction,[4,5,18,19,22,23] but its results were excellent for apple, peach, and cucumber in particular. As with other base-sensitive analytes, chlorothalonil degraded in the least acidic matrices (broccoli and squash), and its partial degradation in the calibration preparation solutions over time affected results for tomato, grape, and green bean. The calibration preparation solutions were kept too long and often in a lighted lab at room temperature in the study, and it was a mistake not to acidify the mixture solutions.[29]

Dichlorvos is the most volatile pesticide added to the bulk samples, and it was partially lost on some days during the evaporation step in the LC method, as evidenced by the comparison of LC vs. GC recoveries. It is not likely lost during the cryogenic comminution step,[30] and like chlorothalonil, it gave higher recoveries in the more acidic fruits (e.g. apple, grape, peach) than the less acidic vegetables (e.g. green bean, broccoli, squash). This points more to degradation than volatilization. Hexachlorobenzene is also relatively volatile, but its consistently ≈70% recovery in all matrices is by design. The choice to use 1 mg CarbonX sorbent dispersed within the ITSP cartridges removes 90% of co-extracted chlorophyll, but also retains 30% of hexachlorobenzene.[24]

Measurement uncertainties

As described in Materials and Methods and previously,[4,5] the RSDs of the results were used to calculate the isolated CV components in the method(s) to estimate measurement uncertainties

with and without use of an int. std. Table 3 contains the calculated CVs for the QC standards that are independent of subsample sizes in the different commodities. A "0" value in the table reflects an imaginary number (*i*) when the measured RSD for a QC standard for a later step in the protocol exceeds the RSD for a previous step. This rarely happened for the QC_{ITSP} calculation because only RO or MeCN standards were used as RSD_{Anal} (CV_{Anal}), but atrazine-d5 (QC_{Extr}) often yielded lower RSD in both the UHPLC-MS/MS and ITSP+LPGC-MS/MS analyses due to its high response factors and few matrix effects.

When peak areas are normalized to atrazine-d5, $CV_{Extr} = 0$ by definition, which is why this factor does not appear in Table 3. However, the contribution of atrazine-d5 to the measurement uncertainty is distributed elsewhere in the calculations, such as the increase of average CV_{Anal} from 3.0% to 4.7% in the case of LC. Despite that normalization to atrazine-d5 corrected an obvious outlier of ^{13}C-phenacetin (QC_{Anal}) in green bean from 19% to 4% RSD, use of the int. std. gave the opposite effect in all other matrices except tomato and apple. In the case of GC, normalization to atrazine-d5 made little difference in the CV_{Anal} because volumes were fixed in that case, but average CV_{ITSP} was improved from 5.2% to 3.3% by better compensating for the elution volume fluctuations in the ITSP step. Otherwise, the error contribution of the QC_{Extr} for the data set using normalized peak areas showed up in higher CV_{Proc} values, as discussed below.

The average CV_{Anal}, CV_{ITSP}, and CV_{Extr} results in Table 3 were used to calculate the estimated measurement uncertainties presented in Figure and Table 4. The latter presents the results for each test portion weight, and the former shows the isolated and overall degrees of precision in the form of pie charts. $CV_{Overall}$ for all test portions and both types of analysis ranged from 7.7% to 10.6%. In both cases, the CV improved by 1-2% overall by not using the int. std., but the actual $RSD_{Overall}$ (RSD_{Proc}) averaged 8.4% in LC whether the int. std. was used or not, and normalization to atrazine-d5 improved overall RSD from 10.5% to 9.3% in the case of ITSP+LPGC-MS/MS. Thus in actual practice, it is probably better to use the int. std. although the results are similar in either case if pipetting is done carefully. Positive displacement pipets were used in this study, which achieve greater accuracy for organic solvents than air-displacement pipets if care is taken to check for and eliminate bubbles.

Including all test portion sizes, average CV_{Proc} ranged from 5.8-9.1% as shown in Figure 4. In theory, CV_{Proc} should be the same in all calculations because sample processing is disassociated from the sample preparation and analysis steps. However, the setting of CV_{Extr} to

559 0 via normalization transfers its degree of uncertainty to the other steps in the method, especially
560 to CV_{Proc} as observable in Figure and Table 4. As also shown in Figures 2 and 3, results in
561 Table 4 demonstrate that 0.5 and 0.25 g test portions lead to noticeably higher inaccuracies than
562 1–15 g. Removal of 0.25 and/or 0.5 g results from the data sets yielded average CV_{Proc} of 5.6-
563 7.5%, $CV_{Overall}$ of 6.9-9.1%, and $RSD_{Overall}$ of 6.6-7.9%. Based on these findings, a 2 g test
564 portion is recommended for routine applications, which was shown to yield on average <9%
565 RSD and no measurable relative biases (Tables 1, 2, S3, and S4 give results for individual
566 analytes, commodities, and test portion amounts).
567 In comparison, the same food processing device at ambient conditions for 1-20 g test
568 portions gave ≈9% CV_{Proc}, which improved to ≈7% when using dry ice and/or a 2-step
569 comminution procedure with a specialized cryomill.[4,5] The original QuEChERS sample
570 preparation method followed by similar (but not the same) ITSP+LPGC-MS/MS and UHPLC-
571 MS/MS analytical methods yielded $RSD_{Overall}$ of 9-15%.[4,5] For the same test portion range >1
572 g, the use of the new one-step LN_2-comminution, sample preparation, and analytical protocol
573 improved both CV_{Proc} and $CV_{Overall}$ by 3-5%. This is an exciting development that others in the
574 field should consider to also validate and implement.
575
576 **Summary of objectives and findings**
577 1) Determine the smallest test portion size subsampled from bulk commodities comminuted
578 in a single step using LN_2 in a conventional food processor: Combined analytical results from
579 28-32 replicates for each among dozens of incurred and spiked pesticides at 7 test portion sizes
580 ranging from 0.25 to 15 g for 8 fruit and vegetable commodities showed that as little as 1 g test
581 portions could be taken without additional measurable bias or precision in the UHPLC-MS/MS
582 and ITSP+LPGC-MS/MS analytical methods. Thus, 2 g test portions weighed into 15 mL
583 tubes for extraction with 10 mL 4/1 (v/v) MeCN/water is a good practical choice for routine
584 analysis without compromising accuracy in the result with respect to the original 500 g bulk
585 sample.
586 2) Devise and implement a safe, practical, and efficient procedure for 1-step comminution of
587 bulk sample commodities using LN_2 with a commercial device: This was accomplished as
588 described in the section on sample processing in Materials and Methods. Working together
589 with one food processor and at least two bowls, two technicians could process 60 fresh

commodity samples in ≈7 hours using the LN_2 procedure, including transfer of comminuted sample to jars for storage and weighing of 2 g test portions into 15 mL tubes for extraction.

3) Test a new method of sample preparation for high-throughput analysis: Using an inexpensive platform shaker (capacity of 100 tubes at once), centrifuge (48 tubes), and evaporator (50 tubes), two technicians could prepare the 60 pre-weighed samples for analysis by both UHPLC-MS/MS and automated ITSP+LPGC-MS/MS in ≈1 hour. A sequence of 75 injections (including 15 QC/calibration standards) in parallel using the fast methods on both instruments takes <19 hours. Use of automated data processing with summation integration yields accurate and reliable "quantidentified" results for hundreds of targeted analytes within a few minutes based on well-established identification criteria, which only entails cursory checking of calibration curves and QC standards by the analyst. Thus, a small team can conduct high-quality start-to-finish processing-to-reporting of hundreds of pesticides in 60 fresh bulk samples within 24 hours by this approach using two instruments and unexceptional laboratory equipment.

4) Test and implement newly re-optimized LPGC-MS/MS conditions for improved analysis: The optimization experiments and choices will be reported separately in the near future, and as shown in this study, <9% RSDs with <1 ng/g LOQs were achieved for commonly monitored pesticides and commodities after automated ITSP cleanup conducted via a standard robotic autosampler in parallel during each analysis. The results for chlorothalonil, for example, showed improved quality vs. previous versions of QuEChERS and GC-MS analysis.

5) Test and implement a rapid sample preparation approach for UHPLC-MS/MS analysis: The new protocol calls for taking 200 µL of the initial aqueous MeCN extract for a ≈5 min evaporation step to just dryness, reconstitution with water, and ultracentrifugation for 5 min. The lack of MeCN in the final extracts improved peak shapes for the first analytes to elute, but further optimization and evaluation is needed for those analytes that elute near the end of the reversed-phase chromatograms. This will be the subject of a future study.

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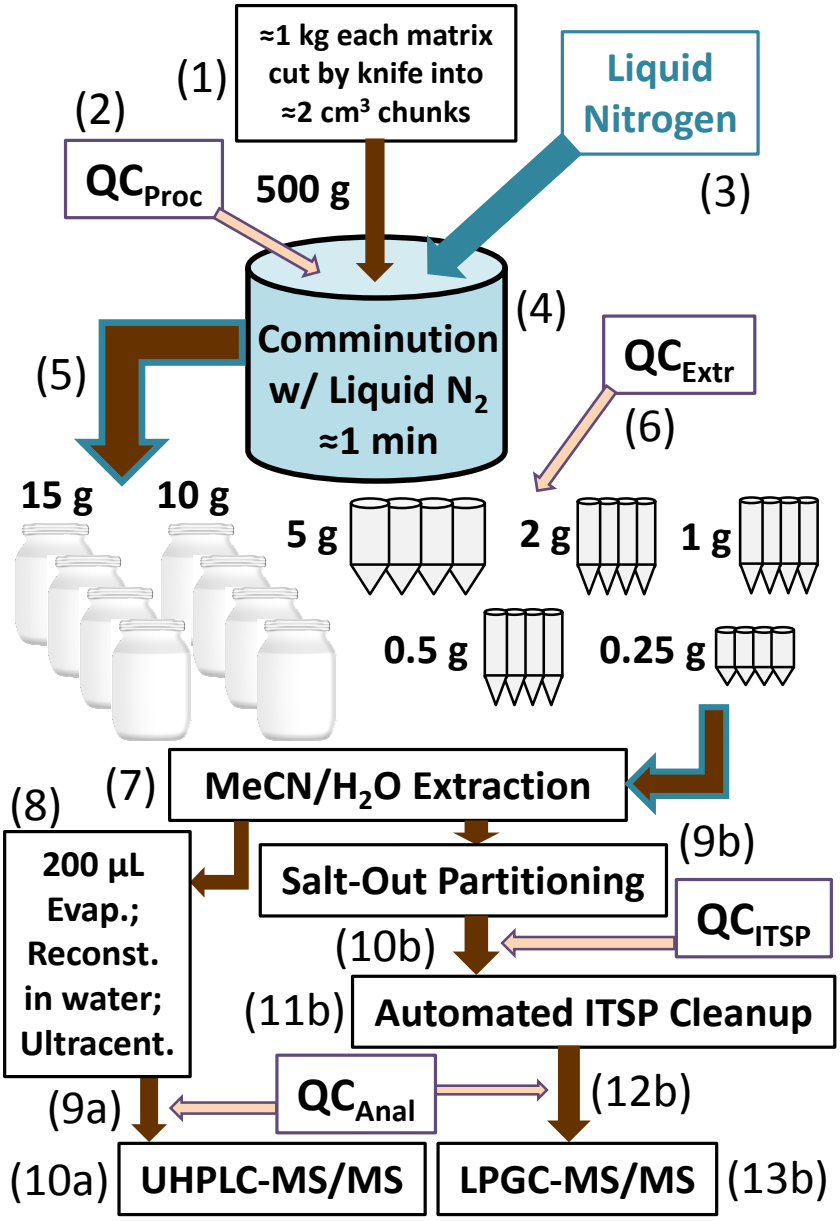

Figure 1. Experimental design of the study.

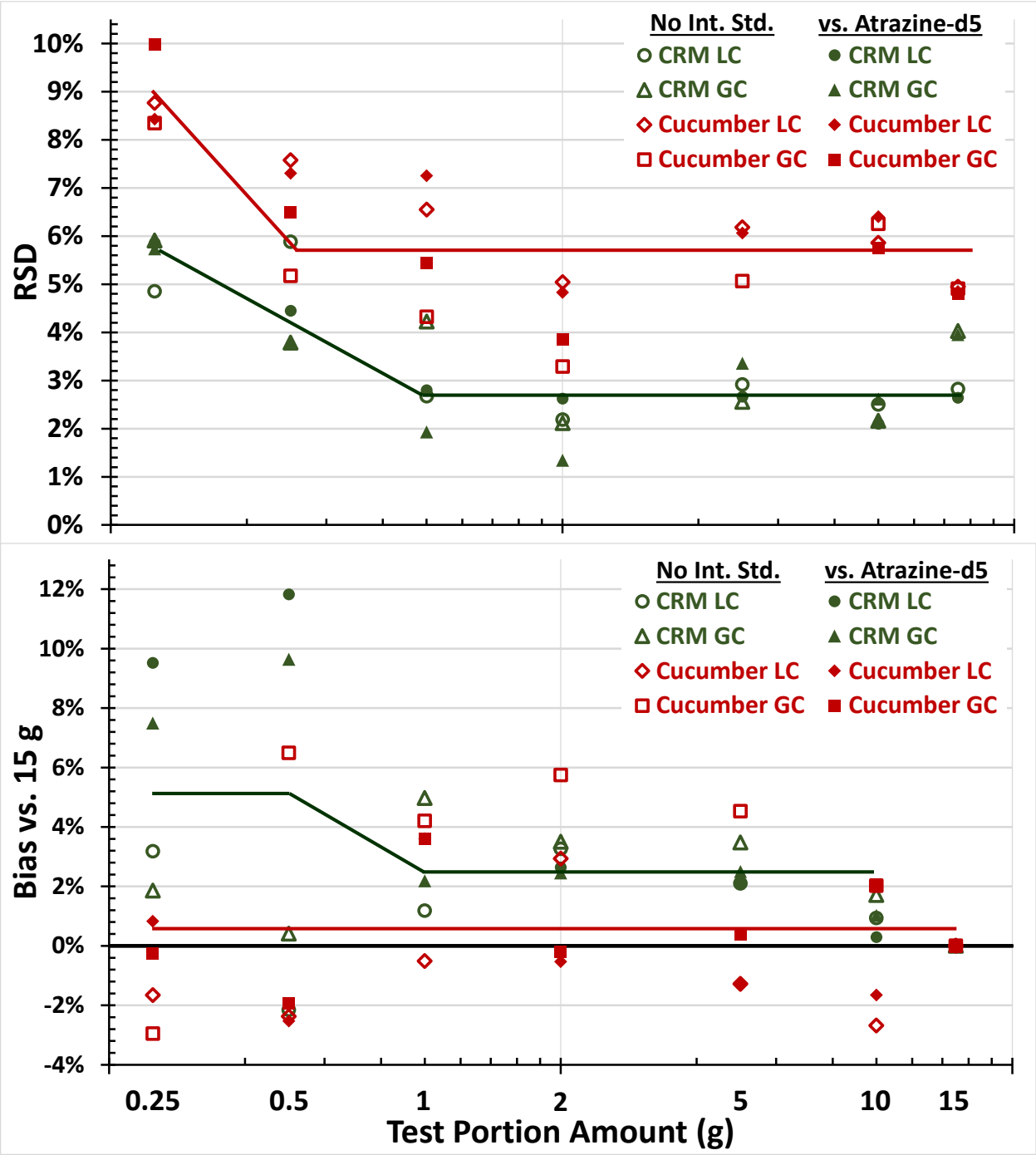

Figure 2. Comparison precision (upper) and bias (lower) in the analysis of the cucumber CRM (no comminution) and store-bought cucumber (LN_2-comminuted) samples plotted vs. test portion size (log axis) with and without normalization of peak areas to atrazine-d5 int. std. Solid lines indicate averages.

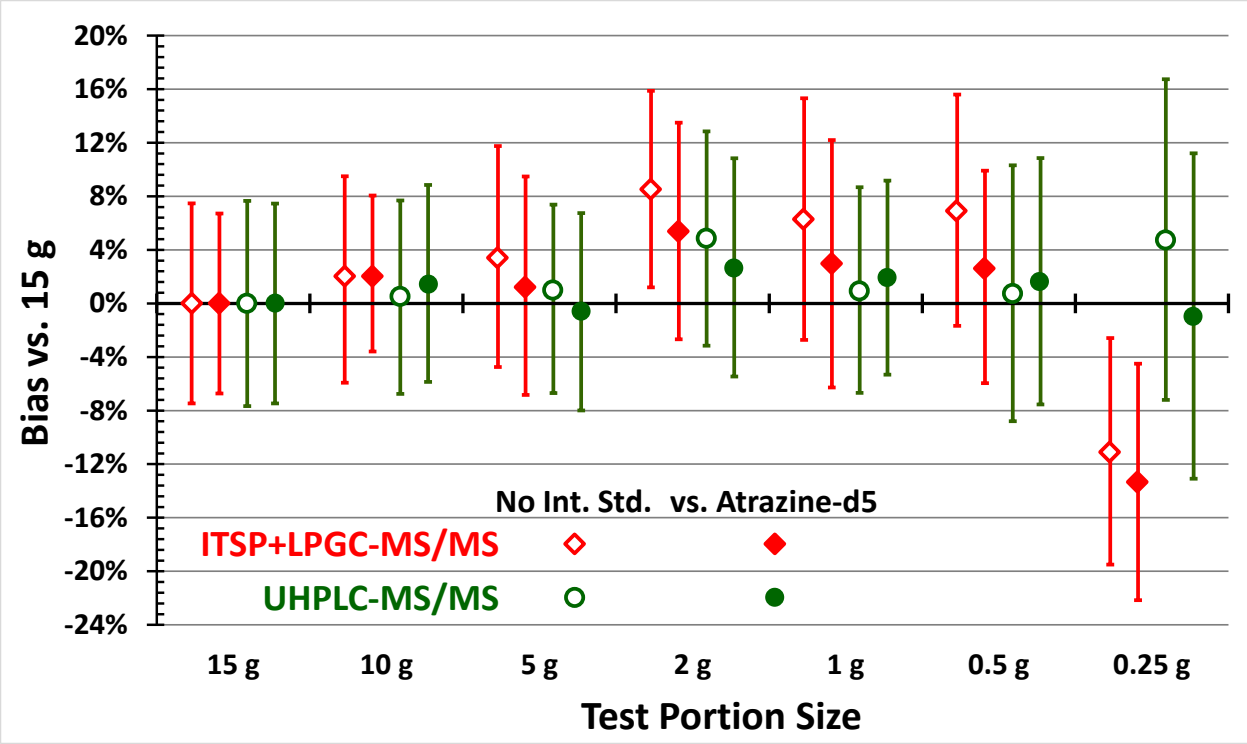

Figure 3. Accuracy (average relative bias and +1 and -1 RSD error bars) in the analyses of all incurred and spiked pesticides in the 8 commodities at the different test portion sizes in ITSP+LPGC-MS/MS and UHPLC-MS/MS with and without normalization of peak areas to atrazine-d5 int. std.

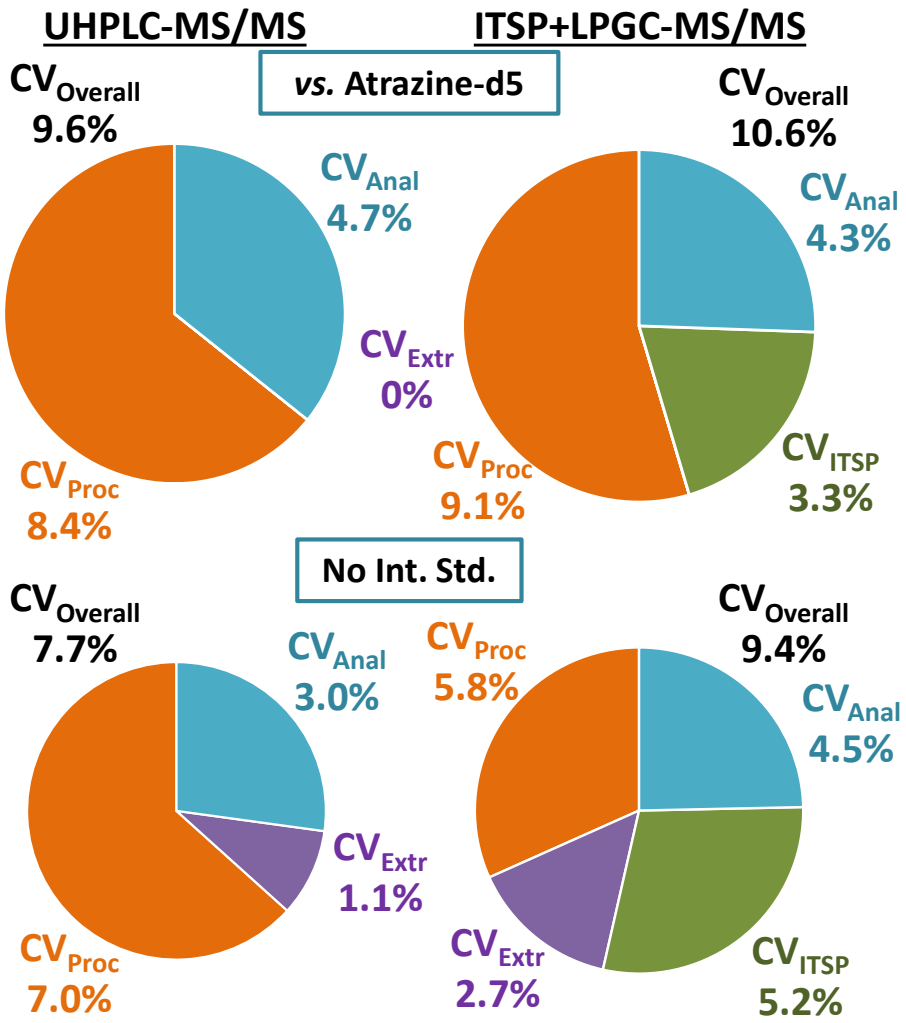

Figure 4. Calculated measurement uncertainties in the study based on averages of the incurred and spiked QC_{Proc}, QC_{Extr}, QC_{ITSP}, and QC_{Anal} analytes in all 8 commodities (and CRM) and both analytical methods with and without normalization to atrazine-d5.

Table 1. Average determined concentrations (ng/g) ± standard deviations (n = 28) of incurred pesticides with peak areas normalized to atrazine-d5 in the different commodities independent of test portion size.

Pesticide	Tomato	Squash	Broccoli	Apple	Grape	Peach	Green Bean	Cucumber	Cucumber CRM
Acetamiprid *	5.3 ± 0.2								91.4 ± 2.2
Azoxystrobin *	26.7 ± 1.9		1.7 ± 0.1	0.8 ± 0.1		0.6 ± 0.1	0.4 ± 0.1		905 ± 24
Azoxystrobin	21.3 ± 3.3		1.5 ± 0.4	1.3 ± 0.5		1.3 ± 0.5	0.6 ± 0.1		900 ± 44
Boscalid *	4.4 ± 0.6				302 ± 28	49.5 ± 5.3			
Boscalid	4.3 ± 0.5				262 ± 26	50.7 ± 5.3			
Carbendazim *									132.0 ± 3.9
Chlorantraniliprole *			2.2 ± 0.3	7.2 ± 0.7		35.2 ± 3.2			
Chlorpropham			4.1 ± 0.3	3.8 ± 0.4					
Chlorpyrifos									92.1 ± 4.6
Cyfluthrin								2.1 ± 0.9	
Cypermethrin									73.8 ± 5.1
Cyprodinil *						345 ± 22			
Cyprodinil						216 ± 18			
p,p'-DDE			3.4 ± 0.7						
Diazinon *									46.7 ± 3.9
Diazinon									61.7 ± 3.3
Difenoconazole *						67.2 ± 9.2			
Difenoconazole						26.7 ± 5.5			
Endosulfan I								0.9 ± 0.2	43.4 ± 2.1
Endosulfan II								0.7 ± 0.2	19.2 ± 1.0
Endosulfan sulfate		1.3 ± 0.1					0.4 ± 0.0	16.6 ± 0.7	
Etoxazole *						24 ± 11			
Fenitrothion									83.5 ± 5.0
Fenbuconazole *						39.6 ± 4.1			
Fenbuconazole						24.0 ± 3.0			
Fludioxonil *				< LOQ		1,300 ± 230			

Pesticide	Tomato	Squash	Broccoli	Apple	Grape	Peach	Green Bean	Cucumber	Cucumber CRM
Fludioxonil				1.2 ± 0.3		1,810 ± 220			
Fluxapyroxad *	18.5 ± 1.7			1.0 ± 0.1		13.9 ± 0.8			
Hexythiazox *						116 ± 52			
Imazalil *				0.8 ± 0.1					42.6 ± 1.4
Imazalil				< LOQ					46.6 ± 2.1
Imidacloprid *		1.0 ± 0.1	4.7 ± 0.3		15.7 ± 0.8				934 ± 24
Indoxacarb				3.5 ± 0.7		35.2 ± 5.0			
Iprodione *									881 ± 57
Iprodione									875 ± 48
Malathion *									70.4 ± 1.7
Malathion									73.0 ± 3.9
Methomyl *							95 ± 15	1.3 ± 0.1	86.1 ± 1.9
Methooxyfenozide *						21.8 ± 2.1			
Myclobutanil*	4.1 ± 0.5								
Myclobutanil	3.2 ± 0.4								
Penthiopyrad *						1.8 ± 0.2	25.2 ± 1.8		
Penthiopyrad						1.9 ± 0.2	16.9 ± 0.8		
Propamocarb *								272.3 ± 8.2	
Propiconazole *						512 ± 30			
Propiconazole						413 ± 33			
Pyraclostrobin *	55 ± 10				304 ± 46	148 ± 17			
Pyraclostrobin	35.0 ± 6.4				144 ± 27	98 ± 22			
Pyrimethanil *				1.1 ± 0.2					
Pyrimethanil				0.9 ± 0.1					
Tebuconazole *					8.4 ± 3.6				90.0 ± 3.5
Tebuconazole					6.0 ± 2.8				83.6 ± 3.5
Tetrahydrophthlamide				365 ± 32					
Thiabendazole*				0.5 ± 0.0					87.1 ± 3.5
Thiabendazole				< LOQ					65.2 ± 4.8

Pesticide	Tomato	Squash	Broccoli	Apple	Grape	Peach	Green Bean	Cucumber	Cucumber CRM
Thiamethoxam *		1.2 ± 0.1						7.0 ± 0.3	
Thiamethoxam		2.5 ± 0.5						4.7 ± 0.7	
Trifloxystrobin *				2.1 ± 0.4	26 ± 10	155 ± 26			
Trifloxystrobin				1.9 ± 0.6	12.3 ± 3.6	69.2 ± 8.2			

* UHPLC-MS/MS results; all other determinations by ITSP+LPGC-MS/MS (n = 24 for tomato)

Table 2. Average %recoveries (%RSDs), n = 28, of the spiked pesticides in the different commodities independent of test portion size. All results normalized to atrazine-d5 (QC_{Extr}), except for atrazine-d5 itself.

Pesticide	Tomato	Squash	Broccoli	Apple	Grape	Peach	Green Bean	Cucumber	Cucumber CRM
QC_{Anal}									
^{13}C-Phenacetin *	92 (3)	95 (3)	89 (3)	94 (3)	97 (5)	86 (3)	92 (7)	96 (7)	98 (2)
p-Terphenyl-d14	104 (12)	94 (10)	95 (8)	98 (10)	100 (10)	101 (9)	106 (8)	106 (9)	111 (11)
QC_{ITSP}									
Carbophenothion	119 (6)	107 (9)	106 (5)	106 (4)	91 (8)	107 (6)	107 (6)	106 (4)	109 (5)
Piperonyl butoxide	120 (7)	109 (9)	107 (5)	110 (4)	90 (8)	108 (7)	109 (6)	104 (4)	109 (5)
Procymidone	108 (6)	103 (9)	103 (5)	105 (4)	91 (8)	103 (6)	105 (6)	106 (4)	111 (4)
QC_{Extr}									
Atrazine-d5*	90 (2)	98 (2)	94 (2)	106 (3)	112 (8)	108 (2)	100 (11)	101 (2)	99 (2)
Atrazine-d5	99 (8)	95 (10)	100 (4)	86 (9)	93 (7)	94 (5)	94 (5)	95 (9)	91 (4)
Fenthion-d6	95 (5)	90 (13)	80 (14)	90 (10)	83 (20)	102 (3)	87 (16)	87 (14)	95 (2)
Pyridaben-d13	106 (8)	89 (**33**)	82 (**38**)	86 (**33**)	79 (**44**)	86 (18)	83 (**37**)	82 (**31**)	95 (2)
QC_{Proc}									**Averages**
Atrazine *	81 (5)	92 (3)	95 (3)	96 (3)	94 (3)	102 (8)	99 (5)	91 (3)	94 (4)
Atrazine	80 (6)	91 (6)	93 (3)	93 (6)	92 (4)	93 (8)	96 (7)	90 (3)	91 (5)
Azinphos-methyl *	105 (12)	98 (12)	118 (13)	88 (12)	98 (9)	98 (10)	104 (11)	88 (9)	100 (11)
Azinphos-methyl	94 (13)	101 (11)	83 (12)	110 (11)	86 (13)	118 (20)	112 (9)	93 (14)	100 (13)

Pesticide	Tomato	Squash	Broccoli	Apple	Grape	Peach	Green Bean	Cucumber	Cucumber CRM
Chlorothalonil	**65** (9)	**37** (19)	**11** (5)	103 (7)	**125** (6)	102 (7)	84 (16)	103 (6)	79 (11)
o,p'-DDD	74 (6)	86 (8)	90 (3)	91 (5)	90 (7)	87 (9)	92 (7)	86 (4)	87 (6)
Diazinon	78 (6)	88 (6)	90 (3)	88 (6)	93 (5)	93 (9)	94 (7)	88 (3)	89 (6)
Dichlorvos *	**25** (**43**)	**50** (8)	**41** (10)	76 (7)	**60** (10)	107 (8)	**35** (18)	**63** (8)	**57** (14)
Dichlorvos	**62** (6)	**59** (12)	**44** (13)	92 (6)	83 (4)	84 (8)	**49** (9)	81 (6)	**69** (8)
Fenthion	72 (8)	73 (8)	**53** (**24**)	89 (6)	85 (6)	87 (10)	82 (8)	**66** (8)	76 (10)
Hexachlorobenzene	**64** (17)	**65** (7)	72 (4)	70 (7)	71 (7)	**68** (12)	74 (8)	**66** (6)	**69** (9)
Lindane	73 (7)	86 (6)	91 (3)	90 (5)	90 (6)	88 (10)	94 (8)	89 (3)	88 (6)
Linuron *	74 (7)	90 (7)	94 (7)	103 (7)	82 (8)	97 (11)	90 (8)	85 (7)	89 (8)
Linuron	89 (9)	96 (7)	100 (4)	96 (9)	91 (7)	99 (10)	96 (8)	90 (12)	95 (8)
Oxyfluorfen	93 (7)	101 (6)	104 (6)	99 (6)	96 (7)	99 (9)	101 (7)	90 (4)	98 (7)
Parathion-methyl	91 (6)	97 (6)	101 (4)	102 (7)	96 (7)	102 (10)	101 (8)	92 (6)	98 (7)
Propargite	85 (5)	93 (5)	87 (5)	120 (7)	91 (7)	95 (8)	98 (7)	90 (4)	95 (6)
Pyridaben	83 (6)	91 (5)	90 (3)	94 (6)	84 (8)	87 (9)	94 (7)	84 (4)	88 (6)
Pyriproxyfen	80 (7)	87 (5)	93 (3)	91 (7)	80 (8)	84 (10)	90 (8)	80 (5)	86 (7)
Tetraconazole *	111 (7)	117 (6)	111 (5)	120 (4)	97 (6)	**122** (9)	**124** (11)	**124** (7)	116 (7)
Tetraconazole	86 (5)	94 (6)	95 (4)	95 (6)	92 (6)	103 (8)	95 (7)	89 (4)	94 (6)
Vinclozolin	82 (7)	93 (6)	95 (3)	94 (5)	96 (5)	96 (9)	96 (7)	91 (3)	93 (6)

* UHPLC-MS/MS results; all other determinations by ITSP+LPGC-MS/MS (n = 24 for tomato)
Bold numbers indicate %recoveries <70 and >120 and/or %RSD >20.

Table 3. %CV for the different QC steps in the analytical methods for the different commodities with peak areas normalized to atrazine-d5 (QC_{Extr}) or not.

	UHPLC-MS/MS			ITSP+LPGC-MS/MS				
	$\%CV_{Anal}$		$\%CV_{Extr}$	$\%CV_{Anal}$		$\%CV_{ITSP}$		$\%CV_{Extr}$
Commodity	**No Int. Std.**	**vs. QC_{Extr}**	**No Int. Std.**	**No Int. Std.**	**vs. QC_{Extr}**	**No Int. Std.**	**vs. QC_{Extr}**	**No Int. Std.**
Tomato	2.1	2.1	0	5.1	8.2	4.7	0	4.6
Squash	1.6	2.4	0.2	5.4	8.5	11	2.6	0
Broccoli	2.5	4.6	0	4.4	2.2	4.5	4.7	0
Apple	3.9	2.0	0	3.8	2.3	1.8	3.2	7.6
Grape	3.3	5.1	7.6	4.3	2.2	10	7.6	0
Peach	3.1	9.4	0	5.0	4.1	6.1	5.1	0
Green Bean	19*	4.0	0	5.6	5.3	4.1	2.9	0
Cucumber	6.1	7.8	0	3.9	4.2	4.2	0	8.2
CRM	1.5	4.7	1.7	2.8	1.6	0	3.8	3.6
Average	**3.0**	**4.7**	**1.1**	**4.5**	**4.3**	**5.2**	**3.3**	**2.7**

*Outlier

Table 4. Average of calculated CV_{Proc} and $CV_{Overall}$ compared with measured $RSD_{Overall}$ (RSD_{Proc}) in both UHPLC-MS/MS and ITSP+LPGC-MS/MS results test portion weight with and without use of atrazine-d5 (QC_{Extr}) as the int. std. (CV_{Proc} vs. QC_{Extr} = RSD_{Proc}).

	Test Portion Size (g):		15	10	5	2	1	0.5	0.25
LC	$\%CV_{Proc}$	No Int. Std.	6.2	5.8	5.6	6.7	6.1	8.2	11
		vs. QC_{Extr}	7.5	7.4	7.4	8.2	7.2	9.2	12
	$\%CV_{Overall}$	No Int. Std.	7.0	6.6	6.5	7.5	6.9	8.8	11
		vs. QC_{Extr}	8.8	8.7	8.7	9.4	8.6	10	13
	$\%RSD_{Proc}$	No Int. Std.	7.7	7.2	7.0	8.0	7.7	9.6	12
GC	$\%CV_{Proc}$	No Int. Std.	4.3	4.3	6.0	4.6	7.1	5.5	7.2
		vs. QC_{Extr}	5.6	4.9	7.2	6.2	7.7	8.0	9.5
	$\%CV_{Overall}$	No Int. Std.	8.5	8.5	9.5	8.7	10	9.2	10
		vs. QC_{Extr}	7.8	7.3	9.0	8.2	9.4	9.7	11
	$\%RSD_{Proc}$	No Int. Std.	7.3	7.5	7.9	7.2	8.8	8.6	9.4